APPENDIX B

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Our reference
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Canadian Patent Application No. 2,876,499
Title: MESENCHYMAL-LIKE STEM CELLS DERIVED FROM HUMAN EMBRYONIC STEM CELLS, METHODS AND USES THEREOF
Applicant: IMSTEM BIOTECHNOLOGY, INC.

RESPONSE TO EXAMINER'S REQUISITION

In response to the Examiner's Requisition dated April 24, 2019, please consider the following:

IN THE DESCRIPTION

Please cancel pages 1, 11-13, 17, 27-30, 32-37, 41, 70, 72, 74, 83 and 84 presently on file for pages 1, 11-13, 17, 27-30, 32-37, 41, 70, 72, 74, 83 and 84 enclosed herewith

IN THE CLAIMS

Please cancel the set of claims presently on file for claims 1-22 enclosed herewith.

IN THE DRAWINGS

Please delete pages 15/31 and 18/31 presently on file and substitute therefore new pages 15/31 and 18/31 submitted herewith.

REMARKS

Claims 1-22 are now pending.

The claims have been amended in order to correspond to claims granted in corresponding U.S. Patent 9,745,551 and claims allowed in corresponding U.S. application 15/656,473.

Regarding the Examiner's rejection of former claims 1-10, 15-58, 60, 61, 64-80 and 82-90 for allegedly being obvious in view of teaching found in the art cited in the International Preliminary Report on Patentability, the Applicant submits that the claims have been limited to subject-matter which has been found to be novel and inventive in corresponding applications and as such, it is believed that similar conclusions should be rendered in Canada. Reconsideration and withdrawal of the Examiner's rejection are earnestly solicited.

The claims have been amended in order to comply with Subsections 36(1) and 36(2) of the *Patent Act*.

Regarding the Examiner's rejection of claims 1-4, 6 and 15 for being indefinite, the Applicant submits that such subject-matter is no longer recited in the set of claims enclosed herewith. Reconsideration and withdrawal of the Examiner's rejection are earnestly solicited.

All the claims are now ending with a period as requested by the Examiner.

The expression "BM-MSC" has been deleted, rendering moot the Examiner's rejection of former claim 5 for allegedly being indefinite.

Former claim 6 has been deleted, rendering moot the Examiner's rejections under Subsection 27(4) of the *Patent Act*.

The expression "decreasing the expression" and "increasing the expression" have been deleted, rendering moot the Examiner's rejection of former claims 7-10 for allegedly being indefinite.

The claims have been amended in order to comply with Subsection 87(3) of the *Patent Rules*, rendering moot the Examiner's rejection directed to former claims 15, 16, 78 and 79.

Former claims 15 and 78 have been deleted, rendering moot the Examiner's rejection under Subsection 27(4) of the *Patent Act*.

Former claims 16 and 79 have been deleted, rendering moot the Examiner's rejections under Subsection 27(4) of the *Patent Act* and Subsection 87(3) of the *Patent Rules*.

Regarding the Examiner's rejection of former claim 17-58 for not comply with Section 84 of the *Patent Rules*, it is believed that the set of claims enclosed herewith, as acknowledged by corresponding U.S. Examiner, is now directed to subject-matter which is fully supported by the present description and which complies with Section 84 of the *Patent Rules*. Reconsideration and withdrawal of the Examiner's rejection are earnestly solicited.

The claims enclosed herewith have been amended in order to be directed to subject-matter which no longer encompasses a method of medical treatment and fully complies with Section 2 of the *Patent Act,* rendering moot the Examiner's rejection of former claims 27-58, 64-67, 71 and 72.

Typographical errors previously identified by the Examiner in former claims 30 and 52 are no longer present in the set of claims enclosed herewith.

The subject-matter recited in the set of claims enclosed herewith is now clear and precise, and it is believed that the Examiner's rejections of claims 61, 69, 70 and 74 under Subsection 27(4) of the *Patent Act* are now moot.

As mentioned previously, typographical errors identified by the Examiner are no longer present in the set of claims enclosed herewith, rendering moot the Examiner's rejection of former claim 75.

Former claim 80 has been deleted, rendering moot the Examiner's rejection under Subsection 27(4) of the *Patent Act*.

The claims have been amended in order to properly be depending on previous claims, rendering moot the Examiner's rejection under Subsection 87(1) of the *Patent Rules* of former claim 87.

Typographical errors identified by the Examiner in former claims 91 and 92 are no longer present in the set of claims enclosed herewith.

Commissioner of Patents
October 3, 2019

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Pages 29, 30, 41, 70, 72 and 74 have been amended in order to identify the trade-marks noted therein.

Incorporations by reference have been deleted from pages 1, 17, 27-29, 32-37 and 84 of the description. No incorporation by reference of documents were identified in pages 30 and 36, line 1.

Regarding the Examiner's objection of the figures under Subsection 82(9) of the *Patent Rules*, pages 11-13 and 83 of the description have been amended in order to no longer refer to characters in red, green and blue.

Figures 15 and 18 have been amended in order to provide drawings which will permit satisfactory reproduction and thus in order to comply with Subsections 82(1) and 82(3) of the *Patent Rules*.

Favourable reconsideration and issuance of a Notice of Allowance are requested. The Applicant submits that the above is a complete and good faith response to the Examiner's Requisition.

The Applicant has no intention of permitting this application to become abandoned. If this application is abandoned for any reason including under Section 73 of the *Patent Act*, Applicant hereby petitions for reinstatement of the application. The Commissioner is hereby authorized to charge payment of the reinstatement fee in the amount set out in item 7, Schedule II for each instance of abandonment and to charge all prescribed fees necessary to reinstate the application using one of the payment methods specified on the accompanying Fee Payment Form.

If it is not possible to reinstate the application, Applicant hereby requests an extension of time pursuant to Section 26 of the *Patent Rules* for each instance of abandonment to provide the Applicant with time to take all steps necessary to reinstate the application. The Commissioner is hereby authorized to charge payment of the extension fee(s) using one of the payment methods specified on the accompanying Fee Payment Form.

If this application is abandoned, the Applicant asks to be notified at the Commissioner's earliest convenience.

Respectfully,

NORTON ROSE FULBRIGHT CANADA LLP/S.E.N.C.R.L., s.r.l.

Norton Rose Fulbright Canada LLP/S.E.N.C.R.L., s.r.l.

Agents for the Applicant(s)

DB/ap

Enclosures: New pages 1, 11-13, 17, 27-30, 32-37, 41, 70, 72, 74, 83 and 84 of the description
New claims 1-22
New pages 15/31 and 18/31 of the drawings

MESENCHYMAL-LIKE STEM CELLS DERIVED FROM HUMAN EMBRYONIC STEM CELLS, METHODS AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATION

The present application claims priority to U.S. patent application serial No. 61/670,787 on July 12, 2102 and U.S. provisional application Serial No. 61/762,961, filed February 11, 2013.

1. INTRODUCTION

The present invention relates to a method of generating mesenchymal stem cells from human embryonic stem cells using a multi-step method of culturing embryonic stem cells comprising culturing embryonic stem cells under conditions sufficient to produce embryoid bodies, culturing the embryoid bodies under conditions to expand hemangio-colony forming cells in the medium comprising the embryoid bodies, and culturing the hemangio-colony forming cells under conditions that induce differentiation into mesenchymal stem cells. Also disclosed are methods of identifying highly immunosuppressive human embryonic stem cell derived mesenchymal-like stem cells. The invention also relates to the human embryonic stem-cell derived mesenchymal stem cells, solutions and pharmaceutical preparations comprising the human embryonic stem cell-derived mesenchymal stem cells, methods of using the human embryonic stem-cell derived mesenchymal stem cells for treatment and prevention of disease, specifically, T cell related autoimmune diseases, and most specifically, multiple sclerosis, and methods of using the human embryonic stem cell-derived mesenchymal stem cells for the delivery of agents across the blood brain barrier and the blood spinal cord barrier. Also provided herein are methods of using hES-MSCs to modulate the immune system, inhibit immune response to individual's self-antigen and repair damaged central nerve systems. Provided herein are compositions comprising hES-MSCs for use in immunomodulation, methods of providing modified MSC with improved immunosuppressive function through modified gene expression. Also provided are methods of using hES-MSC as drug and/or gene delivery system.

Fig. 4 shows the karyotyping of passage-10 hES-MSC derived from the H9- hESC line.

Figs. 5A-C shows the disease scores of EAE mice treated with hES-MSCs prior to the onset of clinical disease. 10^6 hES-MSC or undifferentiated hESC or saline control (PBS) was i.p. injected into the mice 6 days after the EAE inducing immunization. Panel A shows mice injected with hES-MSCs (CT2), panel B shows mice injected with hES-MSCs (MA09), and panel C shows mice injected with hES-MSCs (H9). N=5 mice per group, $***P < 0.001$.

Figs. 6A-F are bar graphs depicting cumulative disease score (panels A-C) and the maximal disease scores (panels D-F) from days 28-32 post immunization for the mice shown in Figure 5. N=5 mice per group,, $**P < 0.01$.

Fig. 7 is a graph of disease scores of EAE mice treated with hES-MSC or saline control (PBS) post-clinical disease onset. 10^6 hES-MSC were i.p injected into mice 18 days post-immunization. N=6 mice per group, *** $P < 0.001$.

Figs. 8A-B show flow cytometric analyses of regulatory T cells ($CD25^+FoxP3^+$) in the CNS of EAE mice treated with saline (PBS) or hES-MSCs derived from hESC line CT2 15 days after immunization.

Figs. 9-F show a bar graph depicting the total numbers of $CD4^+$, $CD8^+$ cells, Th1 $CD4^+$ T cells, and Th17 $CD4^+$ T cells in the CNS of EAE mice treated with saline control (PBS), hESC or hES-MSC on day 15 post-immunization (panels A-D). Panels E-F show the expression of IL-17 and IFN-gamma in $CD4^+$ T cells from PBS or hES-MSC treated EAE mice. N=4 mice per group, $*P < 0.05$, $**P < 0.01$.

Figs. 10A-D show immunohistochemical detection of myelin basic protein (MBP), CD3 for T cells and IBA1 for microglia on lumbar spinal cord cross sections from EAE mice treated with either hES-MSC (panels a and c) or saline (PBS) (panels b and d).

Fig. 11 shows a quantitative analysis of myelin basic protein (MBP) in the spinal cord was performed using relative fluorescent intensity (RFI) measurement of MBP expression in digitally captured spinal cord hemisections. N=4 to 6 mice per group, $**P < 0.02$.

Figs.12A-C show graphs of disease scores of EAE mice treated with saline (PBS), bone marrow derived MSCs (BM-MSC) or hES-MSC prior to the onset of clinical disease. Panel A shows 5 groups of mice treated with either PBS, hES-MSCs (MA09) or BM-MSCs from one of three different sources. Panel B shows mice treated with PBS, BM-MSC or hES-MSC (CT2) prior to clinical disease onset. Panel C shows mice treated with PBS, BM-MSC

or hES-MSC (MA09). For all experiments shown, N=5 mice per group, ***$P < 0.001$ between hES-MSC and any of the three BM-MSC treated groups.

Figs. 13A-D shows the total number of $CD4^+$, $CD8^+$, Th1 or Th17 T cells in the CNS of EAE mice treated with saline control (PBS), BM-MSCs (BM-MSC lines 1, 2 or 3) or hES-MSC. (N=4 mice per group, *$P < 0.05$,** $P < 0.01$).

Figs.14A-E show the qualitative analysis of myelin content in spinal cord cross-sections of EAE mice treated with saline (PBS), BM-MSCs (BM-MSC lines 1, 2, or 3), or hES-MSCs using Fluoromyelin stain and counterstained with DAPI to indicate infiltration of nucleated cells.

Figs. 15A-B show the localization of fluorescently labeled hES-MSC or BM-MSC in spinal cord cryosections (60μm) taken from EAE mice 14 post immunization. Mice received an i.p. administration of GFP^+hESC-MSC or GFP^+BM-MSC or PBS control. Mice were euthanized following the MSC cell administration and immunostained for GFP (to track the injected hES-MSC or BM-MSC cells), CD31 (vascular) and DRAQ5 (ell nuclei). Panel A is parenchymal inflamed venules. Panel B shows meningeal venules. Isosurface rendered 3D reconstruction of the selected ROI (white dotted box) are shown next to the original images for enhanced spatial perspective. The insets show the GFP-DRAQ5 (upper inset image) and isolated GFP (lower inset image) channels separately.

Figs. 16A-B show the proportion of proliferating $CD4^+$ or $CD8^+$ T cells, respectively, co-cultured in vitro with one of two hES-MSCs (MA09 or CT2) or one of three BM-MSC lines (1, 2 or 3) or no MSCs (PBS). T cells were stimulated with the indicated concentration of anti-CD3 antibody and proliferation was measured by CFSE dilution using flow cytometry. T cells and MSCs were mixed at a ratio of 10:1.N=3 replicates per group.

Fig. 17 shows the proliferation of $CD4^+$ or $CD8^+$ T cells co-cultured with BM-MSC, hES-MSC or no MSC (control) and stimulated with 0μg/ml (NC), 0.1μg/ml or 0.3μg/ml anti-CD3 antibody. Flow cytometry histogram plots show the percentage of divided $CD4^+$ or $CD8^+$ T cells with diluted CFSE signal.

Figs. 18A-J depicts intracellular FACS staining of $IFN\gamma^+$ or $IL\text{-}17^+$ naive $CD4^+$ T cells co-cultured with hES-MSC or one of three BM-MSC cell lines (#2,# 3, or #6) or no MSCs (control) and stimulated with TPA/ionomycin stimulation of hES- or BM-MSC incubated with mouse naïve CD4+ T cells, followed by Th1 or Th17 differentiation for 5 days. Data shown are from 1 of 4 independent experiments.

Figs. 19A-B show relative gene expression levels from hES-MSC or BM-MSC as determined by microarray analysis. N = 2, $*P < 0.05$, $**P < 0.01$.

Figs. 20A-F show the expression of IL-6 and IL-10 in 3 individual BM-MSC and 3 individual hES-MSC lines by intracellular FACS staining.

Figs. 21A-D shows the expression of IL-6 by intracellular FACS staining of IL-6 in BM-MSC or hES-MSC (CT2) cultured with IFNγ. NC is negative control.

Figs. 22A-C show the percent of proliferating CFSE labeled $CD8^+$ T cells stimulated with various doses of anti-CD3 antibody and co-cultured with or without one of three BM-MSC lines (#2, #3 or #6) at a ratio of 10:1. Anti-human IL-6 antibody (10 μg/ml) or isotype control (IgGk) was added to the cultures as indicated. N=4 replicates per data point, $**P < 0.01$.

Figs 23A-B show that IL-6 neutralizing antibody (αIL-6) enhances suppression of BM-MSC on CD4 and CD8 T cell proliferation in vitro; NC= T cells cultured without MB-MSC or anti-IL6.

Figs. 24A-J show the proportion of $IFN\gamma^+$ or $IL\text{-}17^+$ $CD4^+$ T cells detected via intracellular FACS staining after TPA/ionomycin stimulation in vitro. hES- or BM-MSC were incubated with mouse naïve $CD4^+$ T cells at a ratio of 1:10 under the Th17 differentiation conditions for 5 days, in the presence or absence of 10 μg/ml anti-human IL-6 antibody.

Fig. 25 shows the clinical disease scores of EAE mice injected with irradiated hES-MSC (Irr-hES-MSC; from MA09), hES-MSCs (from MA09) or saline (PBS). N=5 mice per group, $***P < 0.001$.

Fig. 26 shows immunostaining of luciferase-expressing hES-MSC (CT2). The luciferase expressing hES-MSCs cultured in Petri dish were immunostained with an anti-luciferase antibody and counterstained for nuclei with DAPI.

Figs. 27A-B show the localization of non-irradiated hES-MSC or irradiated (Irr-hES-MSC) expressing D-Luciferin at various days following injection into EAE mice. Images were taken using the Xenogen IVIS 100 system. Non-irradiated (panels A) and irradiated (panels B) luciferase-expressing hES-MSCs (CT2) are shown in the dorsal and ventral images of EAE mice.

Figs. 28A-D show the effect of the GSK3 inhibitor BIO ((2'Z,3'E)-6-Bromoindirubin-3′ -oxime, 6-BIO) on the differentiation of embryoid bodies (EB) from hES

for immunorejection due to mismatch of MHC antigens between MSC and the recipient (Ohtaki *et al.*, 2008; Uccelli and Prockop, 2010a). One hESC line is sufficient to generate hES-MSC at large scale, in an endless supply, and with easy quality control, suitable for industrial production as a potential therapy to treat patients with MS and other T cell-based autoimmune diseases.

The methods for obtaining EB from hES and then dissociating the EB into HB has been previously reported in Lu *et al.* (2007) and Lu *et al.* (2008) as well as in United States Patent Application Publication No. 2012/0027731.

Human hemangio-colony forming cells can be generated from human embryonic stem cells. Such embryonic stem cells include embryonic stem cells derived from or using, for example, blastocysts, plated ICMs, one or more blastomeres, or other portions of a pre-implantation-stage embryo or embryo-like structure, regardless of whether produced by fertilization, somatic cell nuclear transfer (SCNT), parthenogenesis, androgenesis, or other sexual or asexual means.

Additionally or alternatively, hemangio-colony forming cells can be generated from other embryo-derived cells. For example, hemangio-colony forming cells can be generated (without necessarily going through a step of embryonic stem cell derivation) from or using plated embryos, ICMs, blastocysts, trophoblast/trophectoderm cells, one or more blastomeres, trophoblast stem cells, embryonic germ cells, or other portions of a pre-implantation-stage embryo or embryo-like structure, regardless of whether produced by fertilization, somatic cell nuclear transfer (SCNT), parthenogenesis, androgenesis, or other sexual or asexual means. Similarly, hemangio-colony forming cells can be generated using cells or cell lines partially differentiated from embryo-derived cells. For example, if a human embryonic stem cell line is used to produce cells that are more developmentally primitive than hemangio-colony forming cells, in terms of development potential and plasticity, such embryo-derived cells could then be used to generate hemangio-colony forming cells.

Additionally or alternatively, hemangio-colony forming cells can be generated from other pre-natal or peri-natal sources including, without limitation, umbilical cord, umbilical cord blood, amniotic fluid, amniotic stem cells, and placenta.

It is noted that when hemangio-colony forming cells are generated from human embryonic tissue a step of embryoid body formation may be needed. However, given that embryoid body formation serves, at least in part, to help recapitulate the three dimensional

In specific embodiments, biomarkers in a biomarker profile are nucleic acids. Such biomarkers and corresponding features of the biomarker profile may be generated, for example, by detecting the expression product (e.g., a polynucleotide or polypeptide) of one or more markers. In a specific embodiment, the biomarkers and corresponding features in a biomarker profile are obtained by detecting and/or analyzing one or more nucleic acids expressed from a markder disclosed herein using any method well known to those skilled in the art including, but not limited to, hybridization, microarray analysis, RT-PCR, nuclease protection assays and Northern blot analysis.

In certain embodiments, nucleic acids detected and/or analyzed by the methods and compositions of the invention include RNA molecules such as, for example, expressed RNA molecules which include messenger RNA (mRNA) molecules, mRNA spliced variants as well as regulatory RNA, cRNA molecules (e.g., RNA molecules prepared from cDNA molecules that are transcribed in vitro) and discriminating fragments thereof.

In specific embodiments, the nucleic acids are prepared in vitro from nucleic acids present in, or isolated or partially isolated from a cell culture.which are well known in the art, and are described generally, e.g., in Sambrook et al., 2001, Molecular Cloning: A Laboratory Manual. 3.sup.rd ed. Cold Spring Harbor Laboratory Press (Cold Spring Harbor, N.Y.).

5.4.1.1 Nucleic Acid Arrays

In certain embodiments, nucleic acid arrays are employed to generate features of biomarkers in a biomarker profile by detecting the expression of any one or more of the markers described herein. In one embodiment of the invention, a microarray such as a cDNA microarray is used to determine feature values of biomarkers in a biomarker profile. Exemplary methods for cDNA microarray analysis are described below, and in the examples.

In certain embodiments, the feature values for biomarkers in a biomarker profile are obtained by hybridizing to the array detectably labeled nucleic acids representing or corresponding to the nucleic acid sequences in mRNA transcripts present in a biological sample (e.g., fluorescently labeled cDNA synthesized from the sample) to a microarray comprising one or more probe spots.

Nucleic acid arrays, for example, microarrays, can be made in a number of ways, of which several are described herein below. Preferably, the arrays are reproducible, allowing multiple copies of a given array to be produced and results from said microarrays compared

with each other. Preferably, the arrays are made from materials that are stable under binding (e.g., nucleic acid hybridization) conditions. Those skilled in the art will know of suitable supports, substrates or carriers for hybridizing test probes to probe spots on an array, or will be able to ascertain the same by use of routine experimentation.

Arrays, for example, microarrays, used can include one or more test probes. In some embodiments each such test probe comprises a nucleic acid sequence that is complementary to a subsequence of RNA or DNA to be detected. Each probe typically has a different nucleic acid sequence, and the position of each probe on the solid surface of the array is usually known or can be determined. Arrays useful in accordance with the invention can include, for example, oligonucleotide microarrays, cDNA based arrays, SNP arrays, spliced variant arrays and any other array able to provide a qualitative, quantitative or semi-quantitative measurement of expression of a marker described herein. Some types of microarrays are addressable arrays. More specifically, some microarrays are positionally addressable arrays. In some embodiments, each probe of the array is located at a known, predetermined position on the solid support so that the identity (e.g., the sequence) of each probe can be determined from its position on the array (e.g., on the support or surface). In some embodiments, the arrays are ordered arrays. Microarrays are generally described in Draghici, 2003, Data Analysis Tools for DNA Microarrays, Chapman & Hall/CRC.

5.4.1.2 RT-PCR

In certain embodiments, to determine the feature values of biomarkers in a biomarker profile of level of expression of one or more of the markers described herein is measured by amplifying RNA from a sample using reverse transcription (RT) in combination with the polymerase chain reaction (PCR). In accordance with this embodiment, the reverse transcription may be quantitative or semi-quantitative. The RT-PCR methods taught herein may be used in conjunction with the microarray methods described above. For example, a bulk PCR reaction may be performed, the PCR products may be resolved and used as probe spots on a microarray.

Total RNA, or mRNA is used as a template and a primer specific to the transcribed portion of the marker(s) is used to initiate reverse transcription. Methods of reverse transcribing RNA into cDNA are well known and described in Sambrook et al., 2001, supra. Primer design can be accomplished based on known nucleotide sequences that have been

published or available from any publicly available sequence database such as GenBank. For example, primers may be designed for any of the markders described herein. Further, primer design may be accomplished by utilizing commercially available software (e.g., Primer Designer 1.0, Scientific Software etc.). The product of the reverse transcription is subsequently used as a template for PCR.

PCR provides a method for rapidly amplifying a particular nucleic acid sequence by using multiple cycles of DNA replication catalyzed by a thermostable, DNA-dependent DNA polymerase to amplify the target sequence of interest. PCR requires the presence of a nucleic acid to be amplified, two single-stranded oligonucleotide primers flanking the sequence to be amplified, a DNA polymerase, deoxyribonucleoside triphosphates, a buffer and salts. The method of PCR is well known in the art. PCR, is performed, for example, as described in Mullis and Faloona, 1987, Methods Enzymol. 155:335.

PCR can be performed using template DNA or cDNA (at least 1 fg; more usefully, 1-1000 ng) and at least 25 pmol of oligonucleotide primers. A typical reaction mixture includes: 2 .mu.l of DNA, 25 pmol of oligonucleotide primer, 2.5 .mu.l of 10 M PCR buffer 1 (Perkin-Elmer, Foster City, Calif.), 0.4 .mu.l of 1.25 M dNTP, 0.15 .mu.l (or 2.5 units) of Taq DNA polymerase (Perkin Elmer, Foster City, Calif.) and deionized water to a total volume of 25 .mu.l. Mineral oil is overlaid and the PCR is performed using a programmable thermal cycler.

Quantitative RT-PCR ("QRT-PCR"), which is quantitative in nature, can also be performed to provide a quantitative measure of marker expression levels. In QRT-PCR reverse transcription and PCR can be performed in two steps, or reverse transcription combined with PCR can be performed concurrently. One of these techniques, for which there are commercially available kits such as Taqman™ (Perkin Elmer, Foster City, Calif.) or as provided by Applied Biosystems (Foster City, Calif.) is performed with a transcript-specific antisense probe. This probe is specific for the PCR product (e.g. a nucleic acid fragment derived from a gene) and is prepared with a quencher and fluorescent reporter probe complexed to the 5' end of the oligonucleotide. Different fluorescent markers are attached to different reporters, allowing for measurement of two products in one reaction. When Taq DNA polymerase is activated, it cleaves off the fluorescent reporters of the probe bound to the template by virtue of its 5'-to-3' exonuclease activity. In the absence of the quenchers, the reporters now fluoresce. The color change in the reporters is proportional to the amount of each specific product and is measured by a fluorometer; therefore, the amount of each color is

measured and the PCR product is quantified. The PCR reactions are performed in 96-well plates so that samples derived from many individuals are processed and measured simultaneously. The Taqman system has the additional advantage of not requiring gel electrophoresis and allows for quantification when used with a standard curve.

A second technique useful for detecting PCR products quantitatively is to use an intercolating dye such as the commercially available QuantiTect SYBR Green PCR (Qiagen, Valencia Calif.). RT-PCR is performed using SYBR™ green as a fluorescent label which is incorporated into the PCR product during the PCR stage and produces a flourescense proportional to the amount of PCR product.

Both Taqman and QuantiTect SYBR systems can be used subsequent to reverse transcription of RNA. Reverse transcription can either be performed in the same reaction mixture as the PCR step (one-step protocol) or reverse transcription can be performed first prior to amplification utilizing PCR (two-step protocol). Additionally, other systems to quantitatively measure mRNA expression products are known including Molecular Beacons.RTM. which uses a probe having a fluorescent molecule and a quencher molecule, the probe capable of forming a hairpin structure such that when in the hairpin form, the fluorescence molecule is quenched, and when hybridized the fluorescence increases giving a quantitative measurement of gene expression.

5.4.1.3 Northern Blot Assays

Any hybridization technique known to those of skill in the art can be used to generate feature values for biomarkers in a biomarker profile. In other particular embodiments, feature values for biomarkers in a biomarker profile can be obtained by Northern blot analysis (to detect and quantify specific RNA molecules. A standard Northern blot assay can be used to ascertain an RNA transcript size, identify alternatively spliced RNA transcripts, and the relative amounts of one or more genes described herein (in particular, mRNA) in a sample, in accordance with conventional Northern hybridization techniques known to those persons of ordinary skill in the art. In Northern blots, RNA samples are first separated by size via electrophoresis in an agarose gel under denaturing conditions. The RNA is then transferred to a membrane, crosslinked and hybridized with a labeled probe. Nonisotopic or high specific activity radiolabeled probes can be used including random-primed, nick-translated, or PCR-generated DNA probes, in vitro transcribed RNA probes, and oligonucleotides. Additionally, sequences with only partial homology (e.g., cDNA from a different species or genomic DNA

followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, (SDS-PAGE), immunocytochemistry, and the like to determine the amount of protein or proteins of interest present in a sample. One exemplary agent for detecting a protein of interest is an antibody capable of specifically binding to a protein of interest, preferably an antibody detectably labeled, either directly or indirectly.

For such detection methods, if desired a protein from the cell culture to be analyzed can easily be isolated using techniques which are well known to those of skill in the art. Protein isolation methods can, for example, be such as those described in Harlow and Lane, 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press (Cold Spring Harbor, N.Y.).

In certain embodiments, methods of detection of the protein or proteins of interest involve their detection via interaction with a protein-specific antibody. For example, antibodies directed to a protein of interest. Antibodies can be generated utilizing standard techniques well known to those of skill in the art. In specific embodiments, antibodies can be polyclonal, or more preferably, monoclonal. An intact antibody, or an antibody fragment (e.g., scFv, Fab or F(ab').sub.2) can, for example, be used.

For example, antibodies, or fragments of antibodies, specific for a protein of interest can be used to quantitatively or qualitatively detect the presence of a protein. This can be accomplished, for example, by immunofluorescence techniques. Antibodies (or fragments thereof) can, additionally, be employed histologically, as in immunofluorescence or immunoelectron microscopy, for in situ detection of a protein of interest. In situ detection can be accomplished by removing a biological sample (e.g., a biopsy specimen) from a patient, and applying thereto a labeled antibody that is directed to a protein of interest. The antibody (or fragment) is preferably applied by overlaying the antibody (or fragment) onto a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of the protein of interest, but also its distribution, in a particular sample. A wide variety of well-known histological methods (such as staining procedures) can be utilized to achieve such in situ detection.

Immunoassays for a protein of interest typically comprise incubating a sample of a detectably labeled antibody capable of identifying a protein of interest, and detecting the bound antibody by any of a number of techniques well-known in the art. As discussed in more detail, below, the term "labeled" can refer to direct labeling of the antibody via, e.g., coupling (i.e., physically linking) a detectable substance to the antibody, and can also refer to

indirect labeling of the antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently labeled secondary antibody.

The sample can be brought in contact with and immobilized onto a solid phase support or carrier such as nitrocellulose, or other solid support which is capable of immobilizing cells, cell particles or soluble proteins. The support can then be washed with suitable buffers followed by treatment with the detectably labeled fingerprint gene-specific antibody. The solid phase support can then be washed with the buffer a second time to remove unbound antibody. The amount of bound label on solid support can then be detected by conventional methods.

By "solid phase support or carrier" is intended any support capable of binding an antigen or an antibody. Well-known supports or carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material can have virtually any possible structural configuration so long as the coupled molecule is capable of binding to an antigen or antibody. Thus, the support configuration can be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface can be flat such as a sheet, test strip, etc. Preferred supports include polystyrene beads. Those skilled in the art will know many other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

One of the ways in which an antibody specific for a protein of interest can be detectably labeled is by linking the same to an enzyme and use in an enzyme immunoassay (EIA) (Voller, 1978, "The Enzyme Linked Immunosorbent Assay (ELISA)", Diagnostic Horizons 2:1-7, Microbiological Associates Quarterly Publication, Walkersville, Md.; Voller et al., 1978, J. Clin. Pathol. 31:507-520; Butler, J. E., 1981, Meth. Enzymol. 73:482-523; Maggio (ed.), 1980, Enzyme Immunoassay, CRC Press, Boca Raton, Fla.; Ishikawa et al., (eds.), 1981, Enzyme Immunoassay, Kgaku Shoin, Tokyo. The enzyme which is bound to the antibody will react with an appropriate substrate, preferably a chromogenic substrate, in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorimetric or by visual means. Enzymes which can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-

steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate, dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. The detection can be accomplished by colorimetric methods which employ a chromogenic substrate for the enzyme. Detection can also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

Detection can also be accomplished using any of a variety of other immunoassays. For example, by radioactively labeling the antibodies or antibody fragments, it is possible to detect a protein of interest through the use of a radioimmunoassay (RIA) (see, for example, Weintraub, 1986, Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society. The radioactive isotope (e.g., .sup.125I, .sup.131I, .sup.35S or .sup.3H) can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography.

It is also possible to label the antibody with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wavelength, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The antibody can also be detectably labeled using fluorescence emitting metals such as .sup.152Eu, or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriaminepentacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

The antibody also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound can be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in, which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence.

Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

In another embodiment, specific binding molecules other than antibodies, such as aptamers, may be used to bind the biomarkers. In yet another embodiment, the biomarker profile may comprise a measurable aspect of an infectious agent (e.g., lipopolysaccharides or viral proteins) or a component thereof.

In some embodiments, a protein chip assay (e.g., The ProteinChip.RTM. Biomarker System, Ciphergen, Fremont, Calif.) is used to measure feature values for the biomarkers in the biomarker profile. See also, for example, Lin, 2004, Modern Pathology, 1-9; Li, 2004, Journal of Urology 171, 1782-1787; Wadsworth, 2004, Clinical Cancer Research, 10, 1625-1632; Prieto, 2003, Journal of Liquid Chromatography & Related Technologies 26, 2315-2328; Coombes, 2003, Clinical Chemistry 49, 1615-1623; Mian, 2003, Proteomics 3, 1725-1737; Lehre et al., 2003, BJU International 92, 223-225; and Diamond, 2003, Journal of the American Society for Mass Spectrometry 14, 760-765.

In some embodiments, a bead assay is used to measure feature values for the biomarkers in the biomarker profile. One such bead assay is the Becton Dickinson Cytometric Bead Array (CBA). CBA employs a series of particles with discrete fluorescence intensities to simultaneously detect multiple soluble analytes. CBA is combined with flow cytometry to create a multiplexed assay. The Becton Dickinson CBA system, as embodied for example in the Becton Dickinson Human Inflammation Kit, uses the sensitivity of amplified fluorescence detection by flow cytometry to measure soluble analytes in a particle-based immunoassay. Each bead in a CBA provides a capture surface for a specific protein and is analogous to an individually coated well in an ELISA plate. The BD CBA capture bead mixture is in suspension to allow for the detection of multiple analytes in a small volume sample.

In some embodiments the multiplex analysis method described in U.S. Pat. No. 5,981,180 ("the '180 patent"), and in particular for its teachings of the general methodology, bead technology, system hardware and antibody detection, is used to measure feature values for the biomarkers in a biomarker profile. For this analysis, a matrix of microparticles is synthesized, where the matrix consists of different sets of microparticles. Each set of microparticles can have thousands of molecules of a distinct antibody capture reagent immobilized on the microparticle surface and

can be color-coded by incorporation of varying amounts of two fluorescent dyes. The ratio of the two fluorescent dyes provides a distinct emission spectrum for each set of microparticles, allowing the identification of a microparticle a set following the pooling of the various sets of microparticles. U.S. Pat. Nos. 6,268,222 and 6,599,331, and in particular for their teachings of various methods of labeling microparticles for multiplex analysis.

5.4.3 Use of Other Methods of Detection

In some embodiments, a separation method may be used to determine feature values for biomarkers in a biomarker profile, such that only a subset of biomarkers within the sample is analyzed. For example, the biomarkers that are analyzed in a sample may be mRNA species from a cellular extract which has been fractionated to obtain only the nucleic acid biomarkers within the sample, or the biomarkers may be from a fraction of the total complement of proteins within the sample, which have been fractionated by chromatographic techniques.

Feature values for biomarkers in a biomarker profile can also, for example, be generated by the use of one or more of the following methods described below. For example, methods may include nuclear magnetic resonance (NMR) spectroscopy, a mass spectrometry method, such as electrospray ionization mass spectrometry (ESI-MS), ESI-MS/MS, ESI-MS/(MS).sup.n (n is an integer greater than zero), matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS), desorption/ionization on silicon (DIOS), secondary ion mass spectrometry (SIMS), quadrupole time-of-flight (Q-TOF), atmospheric pressure chemical ionization mass spectrometry (APCI-MS), APCI-MS/MS, APCI-(MS).sup.n, atmospheric pressure photoionization mass spectrometry (APPI-MS), APPI-MS/MS, and APPI-(MS).sup.n. Other mass spectrometry methods may include, inter alia, quadrupole, Fourier transform mass spectrometry (FTMS) and ion trap. Other suitable methods may include chemical extraction partitioning, column chromatography, ion exchange chromatography, hydrophobic (reverse phase) liquid chromatography, isoelectric focusing, one-dimensional polyacrylamide gel electrophoresis (PAGE), two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) or other chromatography, such as thin-layer, gas or liquid chromatography, or any combination

thereof. In one embodiment, the biological sample may be fractionated prior to application of the separation method.

In one embodiment, laser desorption/ionization time-of-flight mass spectrometry is used to create determine feature values in a biomarker profile where the biomarkers are proteins or protein fragments that have been ionized and vaporized off an immobilizing support by incident laser radiation and the feature values are the presence or absence of peaks representing these fragments in the mass spectra profile. A variety of laser desorption/ionization techniques are known in the art (see, e.g., Guttman et al., 2001, Anal. Chem. 73:1252-62 and Wei et al., 1999, Nature 399:243-246.

Laser desorption/ionization time-of-flight mass spectrometry allows the generation of large amounts of information in a relatively short period of time. A biological sample is applied to one of several varieties of a support that binds all of the biomarkers, or a subset thereof, in the sample. Cell lysates or samples are directly applied to these surfaces in volumes as small as 0.5 .mu.L, with or without prior purification or fractionation. The lysates or sample can be concentrated or diluted prior to application onto the support surface. Laser desorption/ionization is then used to generate mass spectra of the sample, or samples, in as little as three hours.

5.4.4 Data Analysis Algorithms

Biomarker expression profile of hES-MSC are factors discriminating between clinical grade hES-MSC and non-clinical grade hES-MSC. The identity of these biomarkers and their corresponding features (e.g., expression levels) can be used to develop a decision rule, or plurality of decision rules, that discriminate between clinical grade and non-clinical grade hES-MSC. Specific data analysis algorithms for building a decision rule, or plurality of decision rules, that discriminate between clinical grade hES-MSC and non-clinical grade hES-MSC. Once a decision rule has been built using these exemplary data analysis algorithms or other techniques known in the art, the decision rule can be used to classify a hES-MSC population into one of the two or more phenotypic classes (e.g. a clinical grade or a non-clinical grade hES-MSC). This is accomplished by applying the decision rule to a biomarker profile obtained from the cell culture. Such decision rules, therefore, have enormous value as defining the quality of hES-MSC.

(Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hygro, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147) genes.

5.6 Stem Cell Collection Composition

The stem cell collection composition can comprise any physiologically-acceptable solution suitable for the collection and/or culture of stem cells, for example, a saline solution (e.g., phosphate-buffered saline, Kreb's solution, modified Kreb's solution, Eagle's solution, 0.9% NaCl. etc.), a culture medium (e.g., DMEM, H.DMEM, etc.), and the like.

The stem cell collection composition can comprise one or more components that tend to preserve stem cells, that is, prevent the stem cells from dying, or delay the death of the stem cells, reduce the number of stem cells in a population of cells that die, or the like, from the time of collection to the time of culturing. Such components can be, e.g., an apoptosis inhibitor (e.g., a caspase inhibitor or JNK inhibitor); a vasodilator (e.g., magnesium sulfate, an antihypertensive drug, atrial natriuretic peptide (ANP), adrenocorticotropin, corticotropin-releasing hormone, sodium nitroprusside, hydralazine, adenosine triphosphate, adenosine, indomethacin or magnesium sulfate, a phosphodiesterase inhibitor, etc.); a necrosis inhibitor (e.g., 2-(1H-Indol-3-yl)-3-pentylamino-maleimide, pyrrolidine dithiocarbamate, or clonazepam); a TNF-α inhibitor; and/or an oxygen-carrying perfluorocarbon (e.g., perfluorooctyl bromide, perfluorodecyl bromide, etc.).

The stem cell collection composition can comprise one or more tissue-degrading enzymes, e.g., a metalloprotease, a serine protease, a neutral protease, an RNase, or a DNase, or the like. Such enzymes include, but are not limited to, collagenases (e.g., collagenase I, II, III or IV, a collagenase from Clostridium histolyticum, etc.); dispase™, thermolysin, elastase, trypsin, LIBERASE™, hyaluronidase, and the like.

The stem cell collection composition can comprise a bacteriocidally or bacteriostatically effective amount of an antibiotic. In certain non-limiting embodiments, the antibiotic is a macrolide (e.g., tobramycin), a cephalosporin (e.g., cephalexin, cephradine, cefuroxime, cefprozil, cefaclor, cefixime or cefadroxil), a clarithromycin, an erythromycin, a penicillin (e.g., penicillin V) or a quinolone (e.g., ofloxacin, ciprofloxacin or norfloxacin), a tetracycline, a streptomycin, etc. In a particular embodiment, the antibiotic is active against

Agents, no matter the type and whether for treatment, prevention, or diagnosis, can be conjugated or attached to the hES-MSCs by any method known in the art including but not limited to synthetic extracellular matrix, alginate-poly-L-Lysine encapsulate and/or container.

In certain embodiments, large scale production at industrial level of manufacturing is included in the present disclosure, methods of which are well known in the art. In certain embodiment, the large scale production includes the use of Hyper-STACK 2D culture system and/or Microcarrier 3D bioreactor.

2. Examples

The present invention may be better understood by reference to the following non-limiting examples, which are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed to limit the broad scope of the invention.

6.1 Example 1-Derivation of hES-HB-MSCs

Using a method to derive HB from hESC via EB, mesodermal cells (MP) were enriched and further differentiated into either HB or MSC depending on subsequent culture conditions.

Material and Methods

Four hESC cell lines were used: H9 (derived from WiCell Research Institute) (Thomson et al. (1998)); CT2 (derived from University of Connecticut Stem Cell Core (Lin *et al.* (2010)); MA09 (an FDA approved, clinical-grade cell line derived at Advanced Cell Technology, Inc.) (Klimanskaya *et al.* (2006)); and ES03-Envy (Envy, a GFP-labeled line, derived at ES International) (Costa *et al.* (2005)). These cell lines were cultured on Matrigel™ (BD Biosciences, San Jose, California) and cultured in TeSR1™ medium, (Stem Cell Technologies, Vancouver, Canada), with or without adding of 0.05-0.2μM of BIO (6-Bromoindirubin-3'-oxime (CAS 667463-62-9)).

hESC cells were then differentiated into EB cells and then enriched for HB as previously described (Lu *et al.* (2008); Lu *et al.* (2007)). 50-80% confluent hEs cell on the Matrigel plate were digested with Dispase (1 mg/ml for 5 to 10 minutes) and then washed with EB formation basal medium, HPGM (Lonza, Walksville, Maryland), or STEMLINE I/II Hematopoietic Stell Cell Expansion Medium (Sigma, St. Louis, Missouri), or StemSpan

These cells were replated onto Matrigel-coated plates containing MSC growth medium (Invitrogen). Twenty-four (24) hours later 5-10% of the cells attached to the plate and 9-14 days later, the attached cells fully differentiated into MSC-like cells.

We have also found that by adding GSK3 inhibitor BIO in the feeder free serum free hESC culture can significantly increase the EB and HB formation efficiency. As shown in Figure 28, adding BIO in the mTesr1 medium can increase the size and yield of the EB culture afterwards. As shown in Figure 29, total EB number was increase 3 folds after using BIO in the mTesr1 Medium. As shown in Figure 30, the percentage of CD45 cell differentiation which is an indicator of the hemangioblast differentiation efficiency is also tripled with BIO treatment compare to traditional mTesr1 Medium.

We also found that after differentiation, CD10 expression level varies between different lines of hESC lines. As shown in Figure 31, hES-MSC from MA09 have extreme high level of CD10, but hES-MSC from H9 and CT2 has lower CD10 expression similar to that from BM-MSC. This is confirmed by both microarray and FACS staining.

6.2 Example 2- Further Characterization of hES-HB-MSC Cells

The MSC cells obtained in Example 1 were further analyzed using flow cytometry, immunofluorescence staining, multi-lineage differentiation, and karyotyping.

Materials and Methods

Flow cytometry was performed as described in Example 1.

Immunofluorescence was performed by fixing cells with 4% paraformaldehyde for 15 minutes, and incubating in PBS containing 0.2% Triton™ X-100 (for permeabilization) and 5% goat serum (for blocking). PBS containing 5% goat serum was used to dilute the primary antibodies. The cells were incubated with the primary antibodies at 4°C overnight, followed by washing with PBS for three times. Afterwards, the cells were incubated with fluorochrome-conjugated, corresponding secondary antibodies at room temperature for 30 minutes and washed with PBS for three times. Finally, the cells were examined under fluorescence microscope to capture both phase and fluorescent images.

The G-banded karyotyping of hES-MSC was conducted through an outsourced service at University of Connecticut-Storrs Laboratory.

Microarray Analysis: HumanHT-12 V3 expression BeadChip (illumina) was used for microarray, genomic studio V2011.1 was used for data analysis.

(Stromnes and Goverman, 2006).

Results

As shown in Figure 5, the hES-MSCs derived from the three hESC lines CT2, MA09, and H9 all significantly attenuated the daily disease scores, as well as the cumulative and maximal disease scores (Figure 6) when injected at 6 days or pre-onset of disease, showing a prophylactic effect of the hES-MSCs. Mice injected with CT2 hEScs manifested high disease scores similar to those seen with control mice receiving PBS injection, ruling out the possibility of the effect of human xenograft in mice.

As shown in Figure 7, treatment with hES-MSCs also had a therapeutic effect on mice that have already developed EAE. When injected with hES-MSCs on day 18 post-immunization (right after the disease score peaked in mice), there was a gradual decline of the disease score in hES-MSC-treated EAE mice with an average score of 1.67 at day 30, whereas the PBS-treated EAE mice had an average score of 2.8 at the same day.

6.4 Example 4- Characterization of the Central Nervous System of the EAE Mice Treated with hES-MSCs

The central nervous system of the EAE mice treated with hES-MSCs was further analyzed.

Materials and Methods

Flow cytometry as described in Example 1 was used. Regulatory T cells in the CNS of EAE mice treated with PBS or hES-MSC (CT2) as described in Example 3 were analyzed day 15 post-immunization through FACS analysis of Foxp3 and CD25.

Th1 and Th17 cells from the CNS of EAE mice treated with PBS or hES-MSC (CT2) as described in Example 3 were analyzed day 15 post-immunization by perfusing EAE mice with 20 ml cold PBS through the left ventricle. The brain and spinal cord were harvested from the perfused mice and ground into small pieces. After digestion with collagenase (1 mg/ml) and Dispase (1 mg/ml) for 20 minutes. at 37°C, the tissues were further ground and passed through a 40-μm strainer. Cells were washed and re-suspended in 4 ml of 40% Percoll™ and overlaid onto 5 ml of 70% Percoll. After centrifugation at 2,000 rpm for 20 minutes cells in the inter layer were collected. The cells were then stimulated with 12-Otetradecanoylphorbol-13-acetate (TPA) at 50 ng/ml (Sigma, MO) and ionomycin at 500 ng/ml (Sigma, MO) in the presence of GolgiStop (BD Bioscience, CA) for 6 hours. Cells

6.12 Example 11- Irradiated hES-MSCs Retain Anti-EAE Effect

Mouse embryonic fibroblasts (MEF) are routinely irradiated to stop mitosis without affecting their feeder activity to sustain self-renewal and pluripotency of hESC, as used since the first derivation of hESC lines (Thomson *et al.* (1998)). Based upon this, it was hypothesized that irradiated MSC may also sustain the anti-EAE effect exerted by non-irradiated MSC.

Materials and Methods

hES-MSC, derived from MA09 hESC were irradiated at 80 Gy right before injecting them into EAE mice at 10^6 cells/mouse at day 6 post-immunization as described in Example 2. Disease scoring was done as described in Example 2.

Results

As shown in Figure 25, a decrease of the disease score in the injected mice was found although milder than the decrease caused by non-irradiated hES-MSC. When the dose of hES-MSC was increased to 2 x 10^6 cells/mouse, similar anti-EAE effects were seen between the irradiated and non-irradiated hES-MSC groups

6.13 Example 12- Irradiated hES-MSCs have a Similar Lifespan to the Host Mice

The lifespan of irradiated hES-MSCs *in vivo* was established to determine if the irradiated cells would be effective on EAE.

Materials and Methods

To determine the lifespan of irradiated hES-MSC *in vivo*, CT2 hESC clone with constitutive expression of luciferase in the hESC and their progeny was produced by transducing the cells with a lentiviral vector (Pomper et al. (2009)). The cells were stained with an anti-luciferase antibody and counterstained for nuclei with DAPI and were confirmed to be luciferase positive by fluorescence microscopy (Figure 26).

The luciferase-expressing hES-MSCs (CT2) were irradiated, and non-irradiated and irradiated cells were injected into EAE mice as described in Example 2.

Results

It was found that the irradiated hES-MSC had about the same lifespan of 7-10 days in the mice post-injection as the non-irradiated hES-MSC (Figure 27). These data, together, suggest that irradiated hES-MSC have similar lifespan in the host mice and can achieve

similar efficacy on EAE (when given at doubled dose) compared to non-irradiated hES-MSC, and no tumors are found in the immune-compromised mice transplanted with hES-MSCs.

6.14 Example 14-Qualification procedure for clinical grade hES-MSCs

hES-MSC are characterized through multi-color flow cytometry analyses and immunofluorescence staining using six groups of markers: (1) MSC-specific markers (set 1): CD73, CD90, CD105, , CD146, CD166, and CD44, (2) MSC-specific markers (set 2): CD13, CD29, CD54, CD49E, SCA-1, and STRO-1, (3) hematopoietic stem/progenitor markers: CD45 and CD34, and endothelial cell marker CD31, (4) immunogenic markers: HLA-ABC, HLA-G, CD80, and CD86, (5) cytokines: IL-10, TGFβ, IL-6, IL-12 and TNFα, and (6) pluripotency markers: OCT4, NANOG, TRA-1-60, and SSEA-4. A clinical grade MSC contains >95% of the cells positive for group-1 markers, >80% positive for group 2, <5% for group 3, >80% positive for IL-10 and/or TGFβ, <5% positive for IL-6, IL-12 and TNFα, and <0.001% co-expressing group 6. Heterogeneity and purity of the cells can be tested as described above. The clinical-grade MSC will be compared side-by-side with the preclinical-grade MSC validated in Aim 3.1 as a positive control.

To examine whether the hES-MSC have a consistent cytokine secretion profile, 24 hr condition medium of hES-MSC will be analyzed for secreted cytokines expression using Multiplex System with R&D Fluorokine MAP multiplex Human Cytokine Panel A and TGF-beta 3-plex. All important cytokines that are critical for MSC function will be analyzed simultaneously with only 50-100ul sample needed, including, but not limited to, CCL2, CCL3, CCL4, CCL5, IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17, TNFα, TGFβ, IFNγ, IFNα, IFNβ, GM-CSF, G-CSF, bFGF, CXCL5, VEGF, TPO, CXCL10, CCL11, CD40 ligand, EGF, HGF, IL12A, IL12, IL-13 and/or Leptin.

hES-MSC are also analyzed for: (1) presence of exogenous materials such as endotoxin and residual cytokines/growth factors used to differentiate hES-MSC, and (2) genomic abnormalities (via karyotyping and whole-genome sequencing).

CLAIMS

1. A method for producing human embryonic stem cell-derived mesenchymal stem cells (hES-MSCs), comprising:

   a. culturing human embryonic stem cells in a serum free medium comprising at least one GSK3 inhibitor at a concentration ranging from 0.05 μM to 0.2 μM, wherein the human embryonic stem cells are cultured in the absence of feeder cells;

   b. culturing the cells from step a) in a serum-free medium comprising vascular endothelial growth factor (VEGF) and bone morphogenic protein 4 (BMP4) in an amount sufficient to induce formation of embryoid bodies comprising human hemangio-colony forming cells;

   c. adding at least one growth factor to the culture resulting from step b), wherein the growth factor is in an amount sufficient to expand human hemangio-colony forming cells;

   d. disaggregating the hemangio-colony forming cells resulting from step c) into single cells; and

   e. culturing the single hemangio-colony forming cells resulting from step d) in mesenchymal stem cell ~~a~~ medium containing serum, knockout serum replacement (KOSR), or in a serum-free medium to induce differentiation of the single cells into human mesenchymal stem cells;

wherein at least 90% of the hES-MSCs express CD73, and said hES-MSCs: (i) comprise greater than 95% of cells expressing CD73, CD90, CD105, CD146, CD166, and CD44; (ii) comprise greater than 80% of cells expressing CD13, CD29, CD54, and CD49E; (iii) comprise less than 5% of cells expressing CD45, CD34, CD31 and SSEA4; (iv) express IL-10 and TGFβ; (v) comprise less than 2% of cells expressing IL-6, IL-12 and TNFα; and (vi) comprise less than 0.001% of cells co-expressing OCT4, NANOG, TRA-1-60 and SSEA4.

2. The method of claim 1, wherein the hES-MSCs do not express IL-6, IL12 and TNFα.

3. The method of claim 1, wherein the hES-MSCs express TGF-beta1, TGF-beta2 and IL10.

4. The method of claim 1, wherein the hES-MSCs do not express CCL2, MMP2 and RAGE.

5. The method of claim 1, wherein the hES-MSCs have lower expression of IFNγR1, and IFNγR2 as compared to IFNγR1 and IFNγR2 expression in bone marrow derived mesenchymal stem cells.

6. The method of claim 1, further comprising a step of irradiating the human mesenchymal stem cells.

7. The method of claim 6, wherein the human mesenchymal stem cells are irradiated with gamma-irradiation.

8. The method of claim 1, wherein the hES-MSCs are further modified by genetic modification, epigenetic regulation, small molecule, nutraceutical, natural compound, or antibody treatment.

9. The method of claim 1, further comprising co-culturing the hES-MSCs with hematopoietic stem cells.

10. The method of claim 9, wherein the hematopoietic stem cells comprise bone marrow hematopoietic stem cells, umbilical-cord hematopoietic stem cells, or a combination thereof.

11. The method of claim 1, wherein the GSK3 inhibitor is (2'Z,3'E)-6-Bromoindirubin-3'-oxime (BIO).

12. A method for immunosuppressing T-cells, the method comprising contacting the T-cells with an effective dose of human embryonic stem cell derived mesenchymal stem cells (hES-MSCs), wherein said contacting results in immunosuppressing T-cells and the hES-MSCs are produced by a method comprising the steps of:

(a) culturing human embryonic stem cells in a serum free medium comprising at least one GSK3 inhibitor at a concentration ranging from 0.05 μM to 0.2 μM, wherein the human embryonic stem cells are cultured in the absence of feeder cells;

(b) culturing the cells from step a) in a serum-free medium comprising vascular endothelial growth factor (VEGF) and bone morphogenic protein 4 (BMP4) in an amount sufficient to induce formation of embryoid bodies comprising human hemangio-colony forming cells;

(c) adding at least one growth factor to the culture resulting from step b), wherein the growth factor is in an amount sufficient to expand human hemangio-colony forming cells;

(d) disaggregating the hemangio-colony forming cells resulting from step c) into single cells; and

(e) culturing the single hemangio-colony forming cells resulting from step d) in mesenchymal stem cell medium containing serum, knockout serum replacement (KOSR), or in a serum-free medium to induce differentiation of the single cells into human mesenchymal stem cells,

wherein at least 90% of the hES-MSCs express CD73, and said hES-MSCs: (i) comprise greater than 95% of cells expressing CD73, CD90, CD105, CD146, CD166, and CD44; (ii) comprise greater than 80% of cells expressing CD13, CD29, CD54, and CD49E; (iii) comprise less than 5% of cells expressing CD45, CD34, CD31 and SSEA4; (iv) express IL-10 and TGFβ; (v) comprise less than 2% of cells expressing IL-6, IL-12 and TNFα; and (vi) comprise less than 0.001% of cells co-expressing OCT4, NANOG, TRA-1-60 and SSEA4.

13. The method of claim 12, wherein the hES-MSCs do not express IL-6, IL12 and TNFα.

14. The method of claim 12, wherein the hES-MSCs express TGF-β1, TGF-β2 and IL10.

15. The method of claim 12, wherein the hES-MSCs do not express CCL2, MMP2 and RAGE.

16. The method of claim 12, wherein the hES-MSCs have lower expression of IFNγR1 and IFNγR2 as compared to IFNγR1 and IFNγR2 expression in bone marrow derived mesenchymal stem cells.

17. The method of claim 12, further comprising a step of irradiating the hES-MSCs.

18. The method of claim 17, wherein the hES-MSCs are irradiated with gamma-irradiation.

19. The method of claim 12, wherein the hES-MSCs are further modified by genetic modification, epigenetic regulation, small molecule, nutraceutical, natural compound, or antibody treatment.

20. The method of claim 12, further comprising co-culturing the hES-MSCs with hematopoietic stem cells.

21. The method of claim 20, wherein the hematopoietic stem cells comprise bone marrow hematopoietic stem cells, umbilical-cord hematopoietic stem cells, or a combination thereof.

22. The method of claim 12, wherein the GSK3 inhibitor is (2'Z,3'E)-6-Bromoindirubin-3'-oxime (BIO).

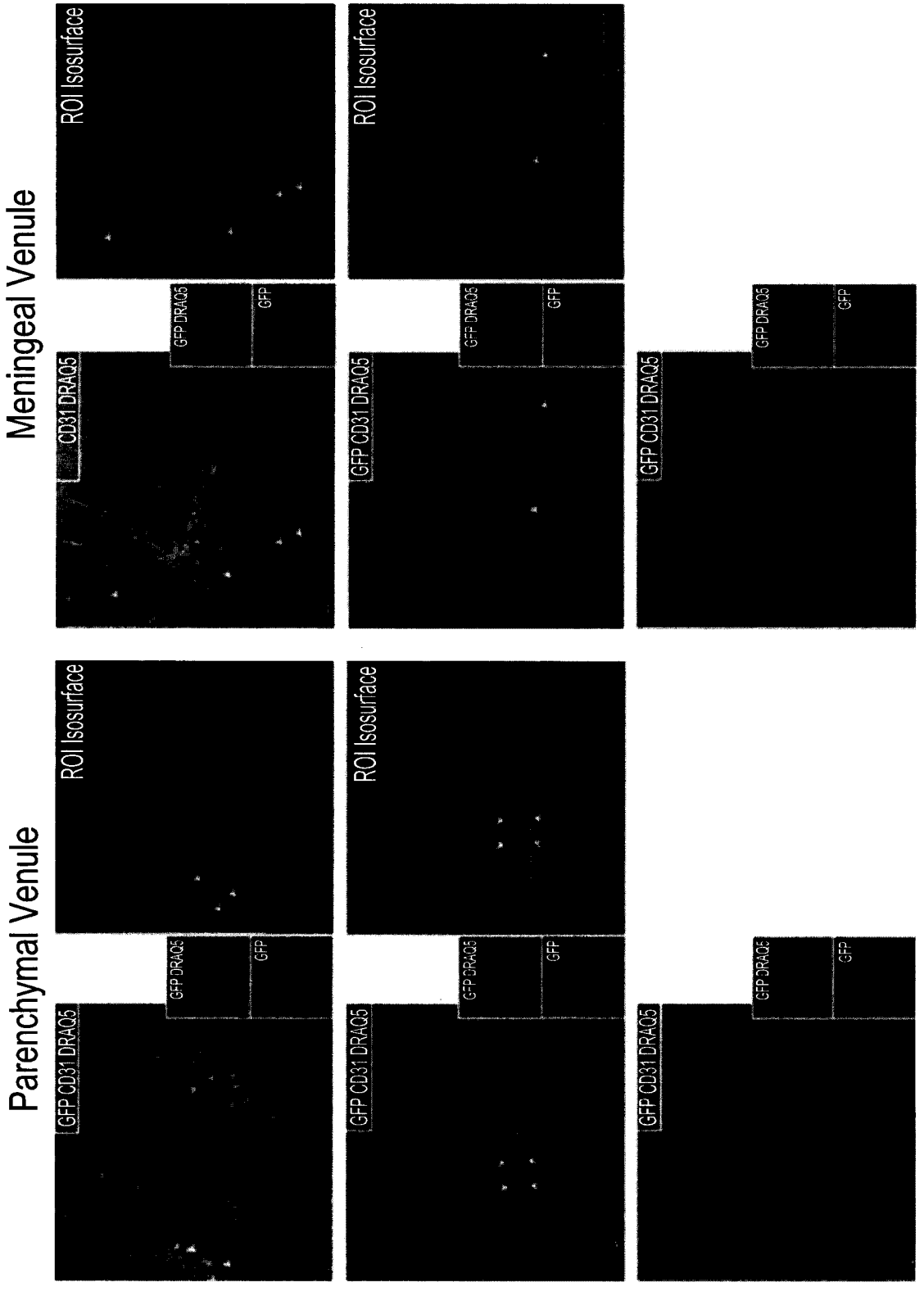

FIG. 15A

FIG. 15B

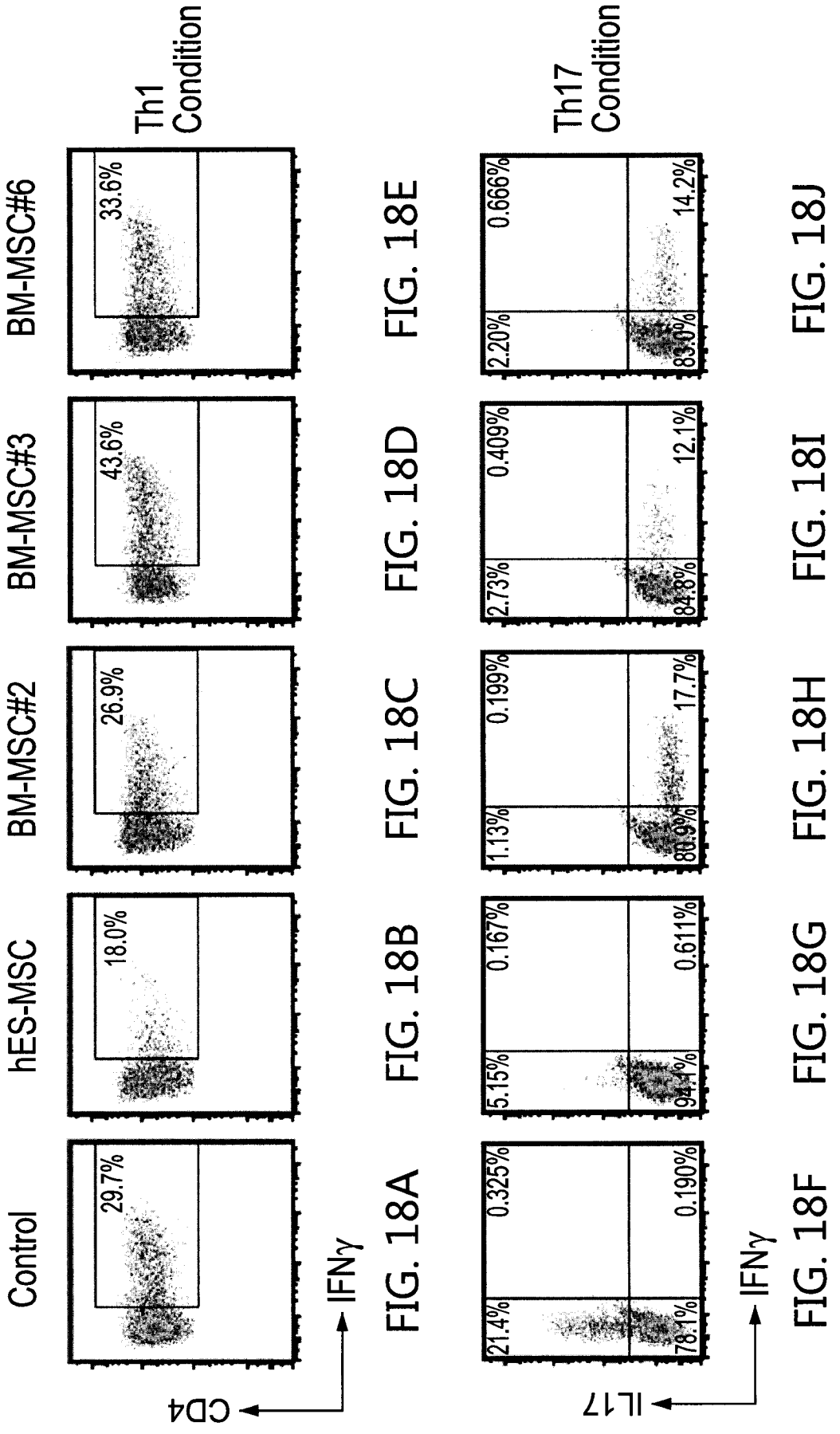

FIG. 18A FIG. 18B FIG. 18C FIG. 18D FIG. 18E FIG. 18F FIG. 18G FIG. 18H FIG. 18I FIG. 18J